

**T and B cell Responses to Multivalent Prime-Boost DNA and Viral Vectors Vaccine  
Combinations against Hepatitis C Virus in Non-Human Primates**

**Running title:** Genetic hepatitis C vaccines in non-human primates

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**Keywords:** hepatitis C virus, vaccines, viral vectors, non-human primate, immunogenicity

## **Abstract**

Immune responses against multiple epitopes are required for the prevention of Hepatitis C Virus infection, and the progression to Phase I trials of candidates may be guided by comparative immunogenicity studies in non-human primates. Four vectors, DNA, SFV, human serotype 5 adenovirus (HuAd5) and Modified Vaccinia Ankara poxvirus (MVA), all expressing HCV Core, E1, E2 and NS3, were combined in three prime-boost regimens, and their ability to elicit immune responses against HCV antigens in rhesus macaques was explored and compared. All combinations induced specific T-cell immune responses, including high IFN- $\gamma$  production. The group immunized with the SFV+MVA regimen elicited higher E2-specific responses as compared to the two other modalities, while animals receiving HuAd5 injections elicited lower IL-4 responses as compared to those receiving MVA. The IFN- $\gamma$  responses to NS3 were remarkably similar between groups. Only the adenovirus induced envelope-specific antibody responses, but these failed to show neutralizing activity. Therefore, the two novel regimens failed to induce superior responses as compared with already existing HCV vaccine candidates. Differences were found in response to envelope proteins, but the relevance of these remain uncertain given the surprisingly poor correlation with immunogenicity data in chimpanzees, underlining the difficulty to predict efficacy from immunology studies.

## Introduction

1 Hepatitis C virus (HCV) vaccine candidates inducing HCV specific immune responses may  
2 protect naïve chimpanzees from heterologous experimental challenge; however the mechanism  
3 behind protection remains elusive<sup>1-6</sup>. Although information regarding the immune correlates of  
4 protective immunity is incomplete, sufficient evidence points towards a pivotal role for a potent  
5 functional cellular immune response against several HCV proteins<sup>7-10</sup>. Vaccine strategies  
6 inducing such strong cellular immunity in mice, rhesus macaques and humans often combine  
7 priming of the immune system with booster immunizations using DNA and recombinant viral  
8 vectors, including adenovirus and modified virus Ankara (MVA)<sup>10,11</sup>.

9 Preclinical evaluation of the potential efficacy of HCV vaccine candidates are limited to the  
10 chimpanzee and humanized mouse models<sup>8,12-14</sup>. Several HCV vaccine candidates have been  
11 created, including viral vectors and typically encoding multiple target antigens, and relied on  
12 preclinical immunogenicity studies in non-human primates such as rhesus macaques<sup>8,15-17</sup>.

13 Comparison of different vectors and regimen in non-human primates are highly valuable for  
14 HCV vaccine development, to shed light on factors specifically influencing HCV vaccine  
15 immunogenicity as well as providing technological improvement in the field of genetic  
16 vaccination, as is performed in HIV development<sup>18</sup>. In a recent study, HCV adenovirus vaccine  
17 boosting induced robust cellular responses, HCV pseudo particles enhanced the humoral  
18 response, and poxvirus priming induced both humoral and cellular responses. Immune responses  
19 were optimized with heterologous prime-boost regimens<sup>16</sup>.

20 Vectors included plasmid DNA, recombinant SFV, replication-incompetent adenovirus and  
21 poxvirus (MVA) encoding the structural HCV antigens Core (C), the two envelope glycoproteins

1 E1 and E2, and the non-structural antigen NS3 were developed and evaluated for  
2 immunogenicity in mice, alone or in various prime-boost combinations. These vectors  
3 successfully elicited cellular and humoral immune responses including in HLA-A2 mice <sup>19</sup>.  
4 Moreover, a DNA-DNA-MVA-MVA vaccine regimen induced robust immune responses  
5 associated with an early control of heterologous HCV infection in chimpanzees<sup>20</sup>. In the present  
6 study, we explored the B and T-cell immune responses induced by this vaccine in rhesus  
7 macaques and investigated if two different immunization regimens, not investigated to date, have  
8 the potential to improve the HCV-specific immune responses: DNA followed by adenovirus, and  
9 SFV followed by MVA. We characterized the immune responses to each of the four vaccine  
10 antigens induced by the 3 regimens in rhesus macaques, and evaluated the predictive value of the  
11 rhesus macaque as a model for HCV vaccine immunogenicity by comparing the immune  
12 response observed in rhesus macaques after DNA-MVA to those observed in chimpanzees after  
13 the exact same DNA-MVA vaccine regimen<sup>20</sup>.

14

1 **Results**

2

3 **All regimens induced lymphoproliferative responses to NS3**

4 Three regimens were tested in three groups of 4 animals (**Fig. 1**). Group I received the DNA  
5 vaccine twice followed by two immunizations with HuAd5, group II received the DNA vaccine  
6 twice followed by two immunizations with MVA, and group III received the rSFV vaccine twice  
7 followed by two immunizations with MVA. HCV-specific lymphoproliferative responses **were**  
8 **tested using recombinant proteins: Core and NS3 from genotype 1a, E1 and E2 from genotype**  
9 **1b**. Responses were induced after the two DNA or SFV injections (**Fig. 2**, individual SI  
10 cumulated for all four antigens **below** 32), and were directed mainly to C (**Fig. 2**, top pie charts,  
11 and supplementary Fig.1), but no significant differences were observed between DNA (both DD  
12 groups) and SFV-immunized (SS) animals.

13 Lymphoproliferative responses were clearly boosted by the HuAd5 or MVA immunizations to  
14 similar high levels (**Fig. 2**, cumulative SI **up to** 168). Analysis of the contribution of each  
15 individual HCV vaccine antigen showed that after Ad or MVA boosting, the NS3-specific  
16 lymphoproliferation dominated, with individual SI ranging from 8 to 55 (**Supplementary Fig. 1**).  
17 The combination SSMM (SFV+MVA) induced significantly higher proliferation to E2 (average  
18 SI of 28) as compared to the other vaccines (average SI of 6 in both DNA-primed groups,  
19  $p=0.04$ , supplementary Fig.1).

20

21 **All regimens induced a Th1-biased T-cell response**

22 We enumerated the C and NS3 (**genotype 1a**), E1, E2 (**genotype 1b**)-induced secretion of IFN- $\gamma$ ,  
23 IL-2 and IL-4, the respective representative Th1 and Th2 cytokines (**Fig. 3**). Although the CD4+

1 cells were not separated from the PBMCs, the ELISPOT assay was performed with recombinant  
2 proteins as stimulus, therefore the responses detected are presumed to represent mainly CD4+ T  
3 cells<sup>21</sup>. After immunization with either DNA or SFV, while lymphoproliferation was detected,  
4 only marginal cytokine production was induced: only 2 animals out of 12 mounting a detectable  
5 IFN- $\gamma$  response, and very low-levels of IL-2 and IL-4 responses were detected (<50 and <34  
6 SFU/million cells respectively against all antigens, data not shown). No difference was observed  
7 between groups (not shown).

8 After the booster immunizations with HuAd5 or MVA, all three regimens induced strong HCV-  
9 specific IFN- $\gamma$  production in all animals, with numbers of specific IFN- $\gamma$  producing cells ranging  
10 from 140 to 1050 SFU/million cells (**Fig. 3a**). There was no statistical difference between the  
11 three groups (**Fig. 3a**). All four antigens contributed to the IFN- $\gamma$  responses (**Fig. 3a**, pie charts,  
12 and supplementary Fig.2), and no statistically significant difference was observed between the  
13 groups, even for the apparent higher C-specific response observed after SFV+MVA injections  
14 ( $p=0.08$ ). The apparent higher E1 response in the group DDAA is also not statistically  
15 significant, due to the high variability of the E1-specific responses in this group ( $p=0.19$ ,  
16 supplementary Fig.2). In contrast, and similarly to the lymphoproliferative response after the two  
17 MVA injections, SSMM induced a significantly higher IFN- $\gamma$  response to E2 (with an average of  
18 95 SFU/million cells) as compared to the other vaccine strategies (with averages of 54 and 10  
19 SFU/million cells in the DDAA and in the DDMM groups respectively,  $p=0.006$ , supplementary  
20 Fig.2).

21 The number of HCV-specific IL-2 secreting cells detected after HuAd5 or MVA injections was  
22 lower than for IFN- $\gamma$ , ranging from 55 to 280 SFU/million cells, and was equivalent between  
23 groups (**Fig. 3b**). All four antigens contributed to the HCV-specific IL-2 response in all three

1 groups (**Fig. 3b**, pie charts, and supplementary Fig.3), however again the SFV+MVA regimen  
2 (SSMM) induced a significantly higher response to E2 ( $p=0.0005$ , supplementary Fig.3).  
3 After HuAd5 or MVA boosts, all animals elicited IL-4 responses, overall lower than IFN- $\gamma$   
4 responses (up to 460 SFU/million cells, **Fig. 3c**). The DNA+HuAd5 regime induced lower IL-4  
5 responses than the two other regimen involving MVA ( $p=0.04$ ), and no response to C. All  
6 vaccine antigens contributed to the IL-4 response (except for C in the DDAA group), and again  
7 the SFV+MVA vaccinated animals elicited significantly higher responses to E2 ( $p=0.01$ ,  
8 supplementary Fig.4). In addition, DNA-MVA induced significantly higher E1 responses than  
9 the other regimens ( $p=0.048$ , supplementary Fig.4).  
10 Taken together, these results demonstrate that the three prime-boost vaccine regimens were able  
11 to induce C, E1, E2 and NS3-specific T-cell responses after the HuAd5 or MVA injections, that  
12 included higher IFN- $\gamma$  production than IL-4 (**Fig. 3d**). Notably, animals receiving two HuAd5  
13 boosts induced lower IL-4 response as compared to the other groups boosted with MVA,  
14 suggestive of a stronger Th1-bias (**Fig. 3d**). The group immunized with the SFV+MVA regimen  
15 elicited higher E2-specific lymphoproliferation and cytokine responses as compared to the two  
16 other vaccine combinations.

17

### 18 **Induction of IFN- $\gamma$ and IL-2 responses to E1 and NS3 peptide pools.**

19 The IFN- $\gamma$  ELISPOT was also performed with overlapping peptides covering E1 and NS3 from  
20 the genotype 1b J strain used in the vaccine vectors. Peptide pools (pp) containing 24 peptides  
21 for E1 (aa 193-377) and 39 peptides for NS3 (NS3pp1 covering aa 1028 to 1346, NS3pp2  
22 covering aa 1340 to 1659) were used. Only 3 animals mounted a detectable E1 or NS3 peptide-  
23 specific IFN- $\gamma$  response after DNA injections, while none responded after the SFV injections

1 (data not shown). The low NS3pp-specific responses in DDAA and DDMM had waned by week  
2 12, 6 weeks post last DNA injection (Fig.4c). In contrast, HuAd5 booster-injections induced E1  
3 peptide-specific responses in all 4 animals (20 to 690 SFU/million cells, Fig. 4a), while MVA  
4 induced lower E1 peptide-specific responses, in 2/4 animals in the DNA+MVA group and in 3/4  
5 animals in the SFV+MVA group (Fig. 4a). Both HuAd5 and MVA boosts induced high NS3-  
6 specific responses in all animals, ranging from 265 to 1525 SFU/million cells for the HuAd5-  
7 induced responses, 340 to 1240 in the DNA+MVA-immunized animals, and 215 to 2180 after  
8 the SFV+MVA regimen (Fig. 4b). The NS3-specific responses were remarkably similar between  
9 groups both in total levels (Fig. 4b), in kinetic (Fig. 4c), and in the contribution of the N-and C-  
10 terminal ends of the protein (Fig. 4d).

11

## 12 **The DDAA vaccine induced superior E2-specific antibody response**

13 The capacity of the three different prime/boost combinations to induce HCV envelope-specific  
14 B-cell responses was analyzed by ELISA using recombinant E1 and E2 proteins from genotype  
15 1b and neutralization assays. Virtually no antibody responses to E1 were detected at any time  
16 point (Fig. 5a). In contrast, E2-specific antibody responses were detected in all three groups  
17 (Fig. 5b). The DNA or SFV injections were poor inducers of antibody responses. After two  
18 HuAd5 injections, strong E2-specific responses were elicited in all 4 animals previously primed  
19 with DNA (Figs 5b and c), while little or no antibody responses were generated in the 8 animals  
20 that received MVA booster injections (Fig. 5c). None of the sera had neutralizing activity in a  
21 E1E2 pseudoparticle assay (data not shown), using pseudoparticles with 100% homology in the  
22 hyper variable region 1 (HVR1 containing the neutralization epitope, Supplementary Fig. 5).

23

## 1 **Discussion**

2

3 In this study, the immunogenicity of three HCV prime-boost vaccine regimens was evaluated in  
4 non-human primates. DNA was compared to SFV immunization, followed by immunizations  
5 with either adenovirus or MVA in rhesus macaques. All vaccine regimens induced Th1 and IFN-  
6 gamma T cell responses. Only the DNA+HuAd5 regime induced antibody responses to the  
7 envelope protein E2, while SFV+MVA induced stronger E2-specific T cell responses.

8 The comparison of DNA and SFV was not conclusive. While lymphoproliferative responses  
9 were induced in all animals, only a limited subset of animals elicited detectable antigen-specific  
10 cytokine responses after DNA or SFV priming. The DNA immunization may not have provided  
11 an optimal priming, as use of specific formulations or delivery devices such as electroporation  
12 would have induced superior priming<sup>15,22</sup>. Notably, higher E2-specific lymphoproliferation and  
13 E2-specific cytokine production were induced after MVA boosting in the group immunized with  
14 SFV+MVA as compared to DNA+MVA, indicating that SFV priming was responsible for this  
15 effect, even though this did not lead to an antibody response to E2 in this group. This could be  
16 due to differences in the constructs rather than the delivery vector: for technical reasons the E2  
17 gene was inserted in a E1-E2 expressing SFV particle as opposed to a combined C-E1-E2 as in  
18 the DNA, adenovirus and MVA vectors. Therefore, differences may be due to different levels of  
19 antigen expression from each vector, or whether the antigens are presented on the cell surfaces.  
20 Combined expression of 3 antigens from the same vector may have compromised independent  
21 expression, or processing or presentation within antigen presenting cells.

22 Other questions addressed by this study included: 1) was HuAd5 “boosting” superior to MVA  
23 after DNA priming and; 2) were rhesus macaque immune responses predictive of the protective

1 HCV responses observed in chimpanzees challenged with HCV? Regarding the boosting  
2 potential of HuAd5, T-cell responses were relatively similar between the DNA+HuAd5 and  
3 DNA+MVA groups. Trends were observed indicating higher proliferation with MVA–boosted  
4 and higher IFN- $\gamma$  responses in the HuAd5-boosted groups, but these were not statistically  
5 significant. The absence of significant differences may be due to the low number of animals per  
6 group, a limitation that is a direct consequence of working with non-human primates. In addition,  
7 the respective immunogenicity of adenovirus and MVA vaccines may be partially antigen-  
8 dependent, as the effect of each antigen immunomodulatory properties may also influence the  
9 cytokine microenvironment induced by the vector. For example, HuAd5 and MVA have been  
10 compared in the context of other antigens: HuAd5 was found superior to poxviruses for  
11 induction of IFN- $\gamma$  T-cell responses to malaria antigens<sup>23</sup>, while MVA encoding the MTB 85A  
12 antigen induces stronger IFN- $\gamma$  producing T-cells after a BCG prime<sup>24</sup>.

13 All three vaccine strategies induced Th1 biased immune responses, with IFN- $\gamma$  responses largely  
14 exceeding the IL-4 responses. A striking difference observed however was the lower IL-4  
15 response in the HuAd5-immunized animals, revealing a more Th-1 bias. However, while MVA  
16 has been shown to induce antibody response to different antigens such as CMV <sup>25</sup>, SARS <sup>26</sup>, Flu  
17 <sup>27</sup>, blood-stage malaria <sup>28</sup>, in this study only the HuAd5 vector induced a strong but transient  
18 antibody response to one of the two envelope proteins. This is in agreement with other recent  
19 HCV vaccine developments demonstrating the capacity of adenovirus boosts to support  
20 envelope-specific antibodies<sup>29</sup>, but confirm that caution should be taken when trying to use  
21 results from different immunogens to influence the design of vaccines for other diseases.  
22 However the antibody response elicited by HuAd5 immunization in our study was not  
23 neutralizing and was short-lived, maybe inducing antibodies masking the neutralization epitopes

1 as recently described <sup>5,30</sup>. The absence of functional activity or persistence of antibodies using  
2 viral vectors was also previously observed <sup>31-33</sup>.

3 This study offers the unique opportunity to compare the immune response elicited by the  
4 DNA+MVA regimen between rhesus macaques and chimpanzees. The exact same constructs and  
5 regimen were used to immunize 4 chimpanzees<sup>20</sup>. In chimpanzees, immunization induced robust  
6 multi-specific immune responses in all four animals, which, following HCV1b exposure, was  
7 associated with a drastic reduction in the peak viremia and RNA levels in liver during the acute  
8 phase of infection. The vaccine regimen induced IFN- $\gamma$  and IL-2 responses as detected by  
9 ELISPOT, with a similar pattern to the one observed in rhesus macaques (strong IFN- $\gamma$   
10 responses and lower IL-2 responses, especially to NS3). Contrary to rhesus macaques however,  
11 the chimpanzees mounted more potent IL-4 responses with numbers of antigen-specific IL-4  
12 producing cells comparable to the number of IFN- $\gamma$  producing cells in 3 out of the 4 animals<sup>20</sup>.

13 The most striking difference between the two primate species was that DNA+MVA regime  
14 induced a robust antibody response in all chimpanzees against both E1 and E2. It is possible that  
15 the stronger IL-4 response observed in chimpanzees supported the more potent B-cell response,  
16 but the reason for the difference is unknown, and the IL-4 response was not absent in rhesus  
17 macaques, just lower than the IFN- $\gamma$  response. This underlines the difficulty to predict the  
18 immunogenicity of a specific vaccine regime based on only one primate species, and to unravel  
19 the relative contribution of cellular and humoral immunity to prevent acute or chronic disease.

20 With the availability of highly efficient antiviral drug treatments able to cure a large proportion  
21 of HCV infections, the relevance and cost of HCV vaccine development is questioned. However  
22 the treatments are extremely expensive, particularly for developing countries, and thus a vaccine  
23 for preventing or treating HCV infection still has an indication in high prevalence countries <sup>34,35</sup>.

1 While the strong restrictions on research involving chimpanzees will restrict efficacy studies,  
2 expensive phase II clinical trials will become the only possibility for evaluating the efficacy of  
3 vaccine candidates. Therefore, studies identifying approaches that are or not optimal, and  
4 investigating the predictive value of non-human primates are highly relevant in this context.

## Materials and Methods

1

### 2 **Animals.**

3 Twelve outbred, purpose bred naïve rhesus macaques (*Macaca mulatta*) imported from China,  
4 seronegative for adenovirus preponderant genus (including serotype 5) and poxviruses (IgG anti  
5 vaccinia virus IgG), were selected following a comprehensive health-check, and were randomly  
6 assigned into 3 groups. The study and all experimental procedures were approved by the local  
7 animal ethical and use committee and were performed in accordance to the Dutch and  
8 international standards for the use of animals in science. Serum and peripheral blood  
9 mononuclear cells (PBMC) were isolated from blood samples collected at regular time points  
10 under sedation using aseptic techniques (Becton Dickinson, Vacutainer systems). Body weight,  
11 temperature, hematology and biochemistry values were monitored at routine intervals.

12

### 13 **Generation of vaccine constructs.**

14 The gene inserts encoding the proteins C, E1, E2 and NS3 were based on genotype 1b, J strain of  
15 HCV (GenBank: D90208.1)<sup>36</sup> for all the vectors used for immunization and described below.

16 All vectors encoding NS3 (DNA, SFV and HuAd5) were described previously<sup>37</sup>. The plasmid  
17 DNA-C-E1-E2, obtained by inserting the C-E1-E2 (aa 1-746) gene sequences into pgWiz (Gene  
18 Therapy System INC, San Diego), was described previously<sup>20</sup>. The SFV-C and SFV-E1-E2  
19 particles were prepared by inserting the corresponding fragments isolated by PCR into an SFV  
20 expression vector, and packaging of the recombinant RNA into SFV particles<sup>38</sup>. Production of  
21 HCV proteins by the rSFV particles was confirmed by immunofluorescence and by *in vitro*  
22 transfection of BHK cells, metabolic labeling (pulse-chase) and immunoprecipitation followed

1 by SDS-PAGE (data not shown). The SFV-NS3, SFV-core and SFV-E1-E2 were mixed for  
2 immunization. The C-E1-E2 expressing human type 5 replication-defective adenovirus (HuAd5-  
3 C-E1-E2) was described earlier <sup>39</sup>. Human adenovirus serotype 5 was chosen as a prototypic  
4 adenovirus for this study. The recombinant MVA-C-E1-E2, and MVA-NS3 vectors were both  
5 constructed by transient host range selection using the HCV-1b structural (amino acids, aa 1-  
6 830) and nonstructural 3 (aa 1028-1658) genes respectively, as previously described<sup>20</sup>. **All**  
7 **vectors used for immunization and their transgenes are described in Fig.1.**

8

### 9 **Peptides and recombinant proteins used for *in vitro* assays.**

10 The C polypeptide (a.a. 1-120) and NS3 helicase (NS3h, aa 1193-1458), derived from HCV  
11 genotype 1a <sup>40</sup>, were expressed in *E. coli* and purified on Ni-NTA column as described  
12 previously <sup>39</sup>. The envelope proteins E1 and E2, deleted from their transmembrane domain, were  
13 derived from the HCV-1b sequence cloned into pT-alpha vector, and were described  
14 previously<sup>20</sup>. Fifteen-mer peptides, with overlaps of 7 amino acids covering the C, E1, E2 and  
15 NS3 sequences (genotype 1b, J strain <sup>36</sup>), were purchased from Clonestar Biotech (Brno, Czech  
16 republic).

17

### 18 **Immunizations**

19 The immunizations with DNA or rSFV consisted of 2 injections at weeks 0 and 6, and HuAd5  
20 and MVA were administered at weeks 14 and 20 (**Fig. 1**). For each DNA immunization, 2 mg  
21 DNA-C-E1-E2 and 2 mg DNA-NS3 dissolved in saline buffer were equally divided and  
22 administered both intramuscularly (IM) and intradermally (ID). For each SFV immunization,  
23  $5 \times 10^9$  pfu of each construct dissolved in saline were injected subcutaneously (SC). Animals from

1 group I were boosted SC twice with  $5 \times 10^{10}$  pfu of each HuAd5 construct. Animals from groups  
2 II and III were boosted twice with  $5 \times 10^8$  pfu MVA of each construct, again administered both  
3 IM and ID. The constructs were not mixed, the C, E1 and E2 constructs were injected on the left  
4 side and the NS3 on the right. Therefore, the vaccine vectors have been administered via  
5 different routes: the route for each vector was selected to induce the strongest immune response.  
6 Because routes may impact on magnitude and quality of resulting immune responses, the aim of  
7 this study was not to compare the vector's potency by a single route, but to identify the optimal  
8 vaccine regimen, with each vector injected by its optimal route.

9

#### 10 **Analysis of the humoral immune responses**

11 Quantification of anti-adenovirus antibodies was performed in frozen serum by an independent  
12 hospital laboratory (Erasmus MC – Virology, Rotterdam, Netherlands), with a quantitative  
13 enzyme immunoassay (SERION ELISA *classic* Adenovirus IgG/IgA), detecting antibodies to  
14 eight preponderant genus specific epitopes.

15 Anti-HCV antibody responses in frozen sera were measured using ELISA with HCV C (0.5  
16 mg/ml), E1 (4  $\mu$ g/ml), E2 (1  $\mu$ g/ml) or NS3-helicase proteins (0.5 mg/ml). ELISA was  
17 performed as described previously<sup>20</sup>. For each experiment, the cut off was determined as the  
18 mean value plus three times the standard deviations obtained with serum of three random naïve  
19 serum samples.

20 The capacity of the frozen sera to neutralize HCV was analyzed using HCV pseudo-particles  
21 with E1E2 glycoproteins of strain CG1b in infection assays on Huh-7 target cells as previously  
22 described<sup>41</sup>. **Control neutralizations were performed using pseudo-particles generated with**  
23 **glycoproteins derived from the feline endogenous retrovirus RD114 (RD114pp).**

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**Analysis of the cellular immune responses by lymphoproliferation and ELISPOT.**

Lymphoproliferation was measured by <sup>3</sup>H-thymidine incorporation as previously described, using fresh PBMCs <sup>42</sup>. The results were expressed as stimulation index (SI), calculated as cpm with antigen divided by cpm with medium alone. Lymphoproliferation was considered positive when the SI exceeded 2. Quantification of specific cytokine secreting cells was performed by IFN- $\gamma$ , IL-2 and IL-4 ELISPOT assays using fresh PBMCs according to the manufacturer's instructions (U-Cytech, Utrecht, Netherlands), using concanavaline A (ConA), or recombinant proteins or peptides (5  $\mu$ g/ml), or medium alone. The results were expressed as mean spot forming units (SFU) of triplicate assay per one million PBMCs minus the mean number of spots obtained with the cells cultured in medium alone (also in triplicate assays). Results superior to 5 SFC/million PBMCs were considered a positive response.

**Statistical analysis.**

To compare the immunogenicity of the different vaccine combinations, ANOVA, with p values calculated by exact methods and two-tailed, was used. Difference was considered statistical significant when  $p \leq 0.05$ .

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## Figure legends

**Fig. 1. Immunization schedule.** Three groups of four macaques per group were immunized at the four time-points indicated by arrows (at weeks 0, 6, 14 and 20), with the vaccine immunogens as indicated, further referred to as DNA+HuAd5 (DDAA), DNA+MVA (DDMM) and SFV+MVA (SSMM). The composition of vectors and their transgenes used for each immunization (DNA, HuAd5, MVA or SFV) is indicated.

**Fig. 2. Lymphoproliferation responses induced against all four HCV proteins (graph) and the proportional contribution of each antigen (pie charts) after priming and boosting.** The cumulated SI to all four HCV antigens of each animal is shown, each diamond representing one animal, 2 weeks after the two DNA or SFV injections, and 2 weeks after HuAd5 or MVA injections. Individual values mentioned in the text are indicated. The geometric mean + SD of each group at both time-points are also represented as horizontal bars. The contribution of each antigen-specific lymphoproliferation is represented on top of each vaccine regimen, each pie chart representing the geometric mean lymphoproliferation of each group to each antigen as indicated in the legend. Lymphoproliferation was performed using recombinant proteins.

**Fig. 3. Enumeration of IFN- $\gamma$  (a), IL-2 (b) and IL-4 (c) producing cells by ELISPOT against all four HCV proteins (graphs) and the proportional contribution of each antigen (pie charts) after HuAd5 or MVA-booster injections.** (a, b and c) The cumulated HCV-specific Spot Forming Units /million cells per animal are shown after two HuAd5 or MVA injections as indicated, each diamond representing one animal. The mean + SD of each group are also

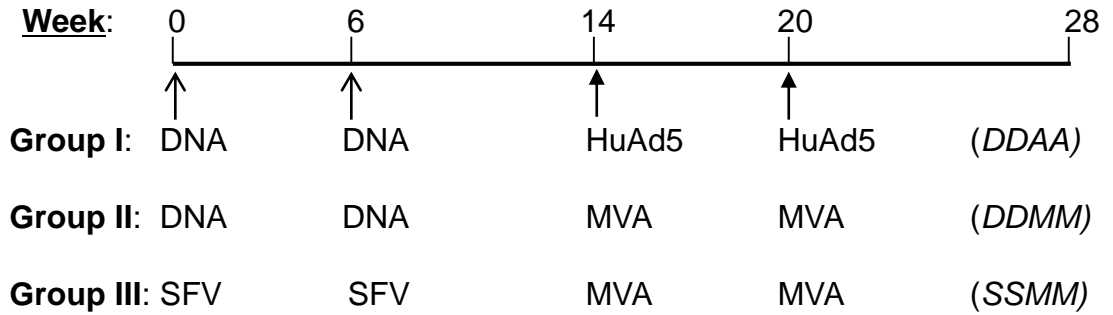
represented as horizontal bars. Some individual values (black) and average group values (red) are indicated for ease of comparison. \* indicates statistical significant difference by ANOVA. The contribution of each antigen-specific cytokine production is represented on top of each regimen, each pie chart representing the mean of IFN- $\gamma$  (a), IL-2 (b) and IL-4 (c) producing cell number of the group to the corresponding antigen as indicated in the coloured legend. (d) Pie charts of the geometric mean cytokine-producing cells showing the contribution of each cytokine responses to all 4 antigens for each regimen. The ELISPOT assays were performed using recombinant proteins.

**Fig. 4. Individual E1 (a) and NS3 (b, c and d) peptide pool-specific IFN- $\gamma$  production by PBMCs.** IFN- $\gamma$  production to E1 or NS3 peptide pools as tested by ELISPOT are represented for each animals from each group post HuAd5 or MVA immunization (a and b respectively). Results are expressed as mean number of spots of triplicate assay per one million cells minus the mean number of spots obtained with the medium cultured cells (also in triplicate assays) + 2SD. (c) the time course of NS3-specific response is shown for the 3 groups as indicated in the legend, as the geometric mean numbers of cytokine producing cells per group. Grey arrows indicate the timing of DNA or SFV injections, and black arrows indicate the timing of HuAd5 or MVA injections. (d) Individual responses to NS3pp1 covering aa 1028 to 1346 and NS3pp2 covering aa 1340 to 1659 after HuAd5 or MVA immunizations are shown, along with the geometric mean of the group.

**Fig. 5. Serum antibody responses to E1 and E2 recombinant proteins.** The geometric mean titers + SD of the E1 (a) and E2 (b)-specific antibody responses in each of the three groups is

represented over time. Black arrows indicate the timepoints of immunizations (DNA or SFV at weeks 0 and 6, HuAd5 or MVA at weeks 14 and 20). (c) Individual responses to E2 at week 22, each dot representing one animal and the horizontal lines representing the geometric mean of the group. Differences of statistical significance between groups are indicated by \*. ELISA were performed with recombinant proteins.

Figure 1



**Constructs used for immunizations:**

**DNA:** DNA-NS3 + DNA-C-E1-E2

**HuAd5:** HuAd5-NS3 + HuAd5-C-E1-E2

**MVA:** MVA-NS3 + MVA-C-E1-E2

**SFV:** SFV-NS3 + SFV-C + SFV-E1-E2

Figure 2

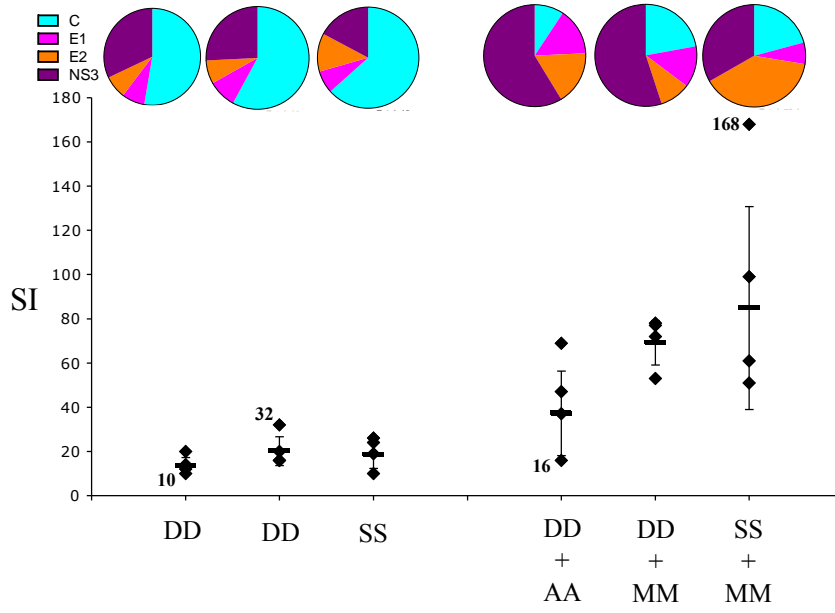


Figure 3

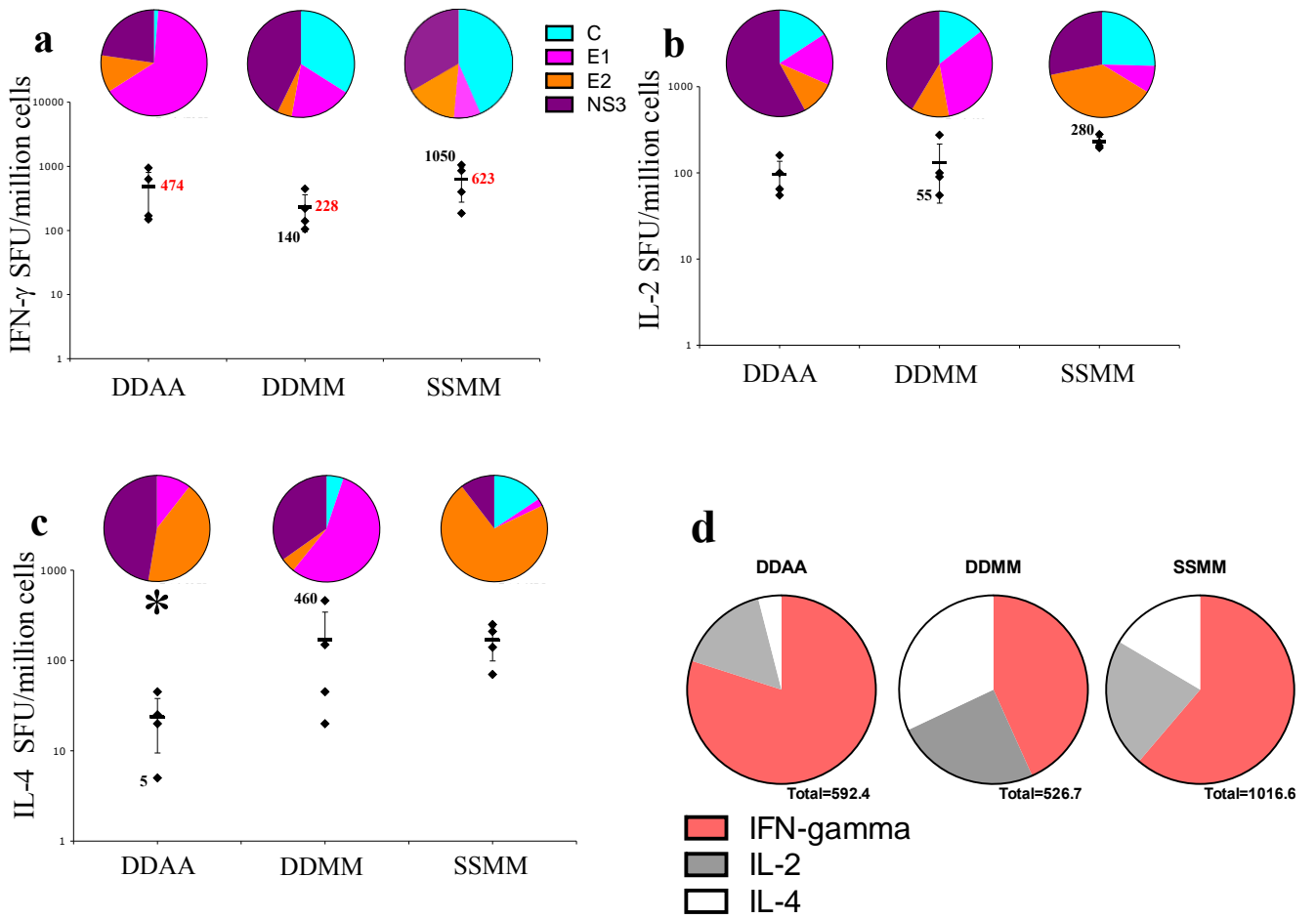


Figure 4

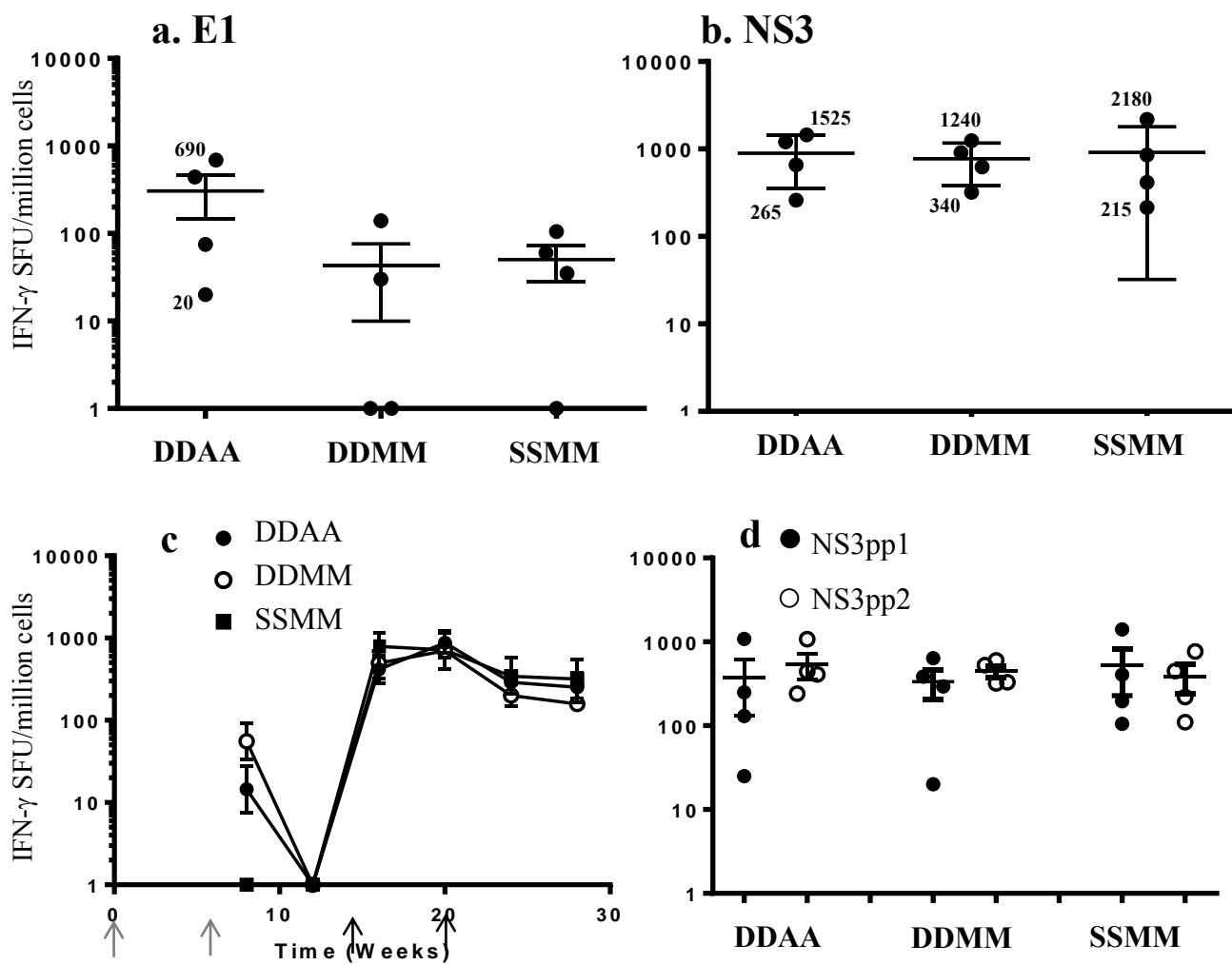
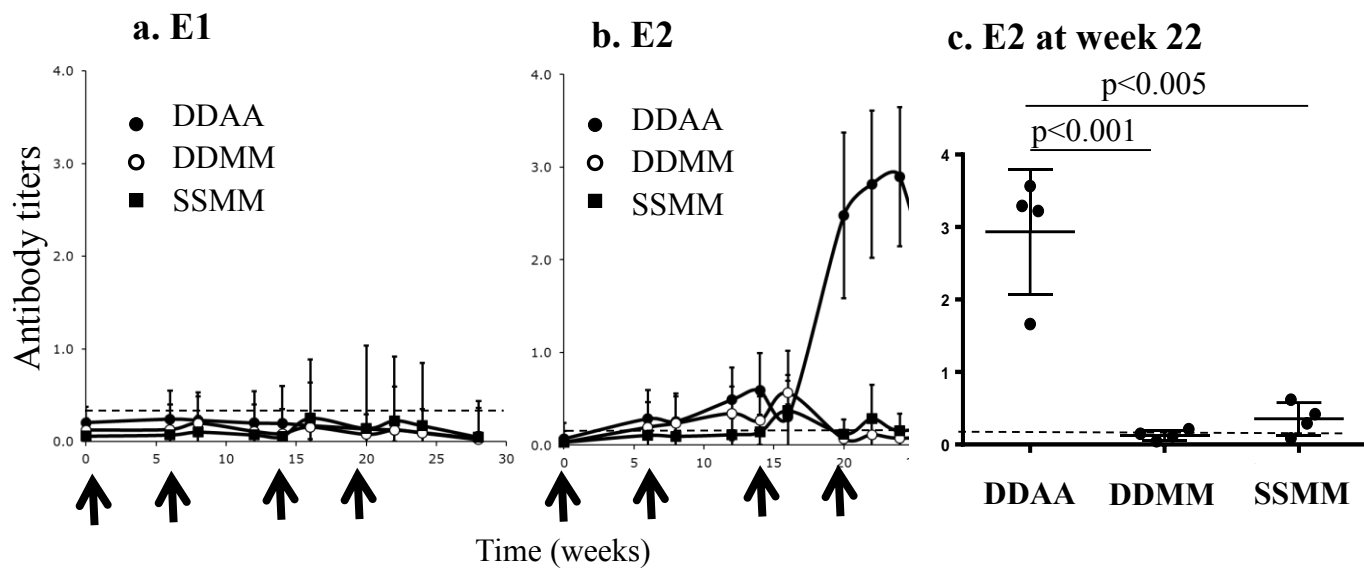
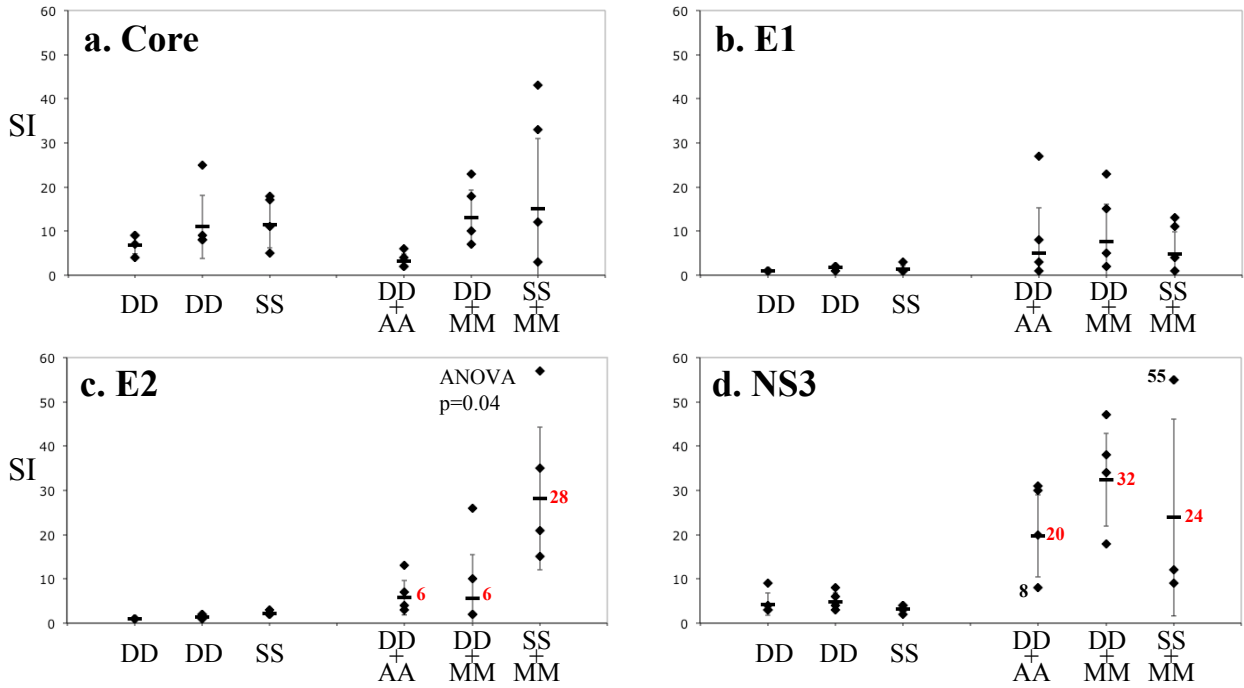


Figure 5



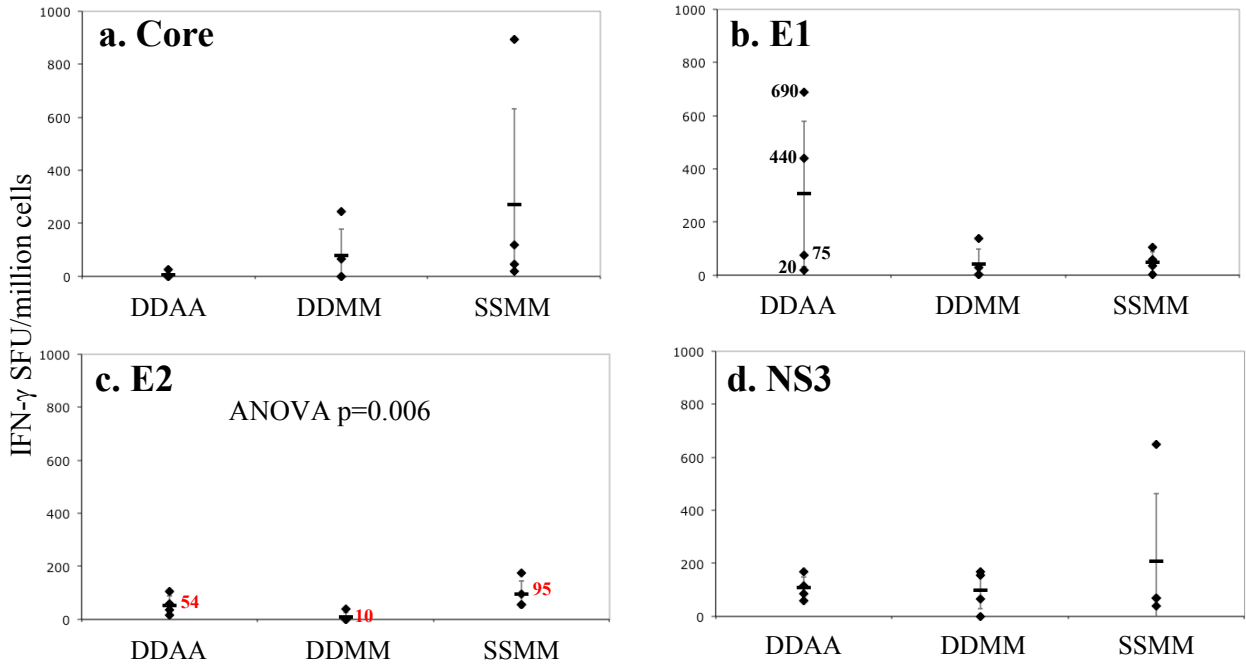
## Supplementary Figure 1

Individual lymphoproliferation to each HCV antigen. Some individual (black) and average (red) values mentioned in the text are indicated for ease of reading.



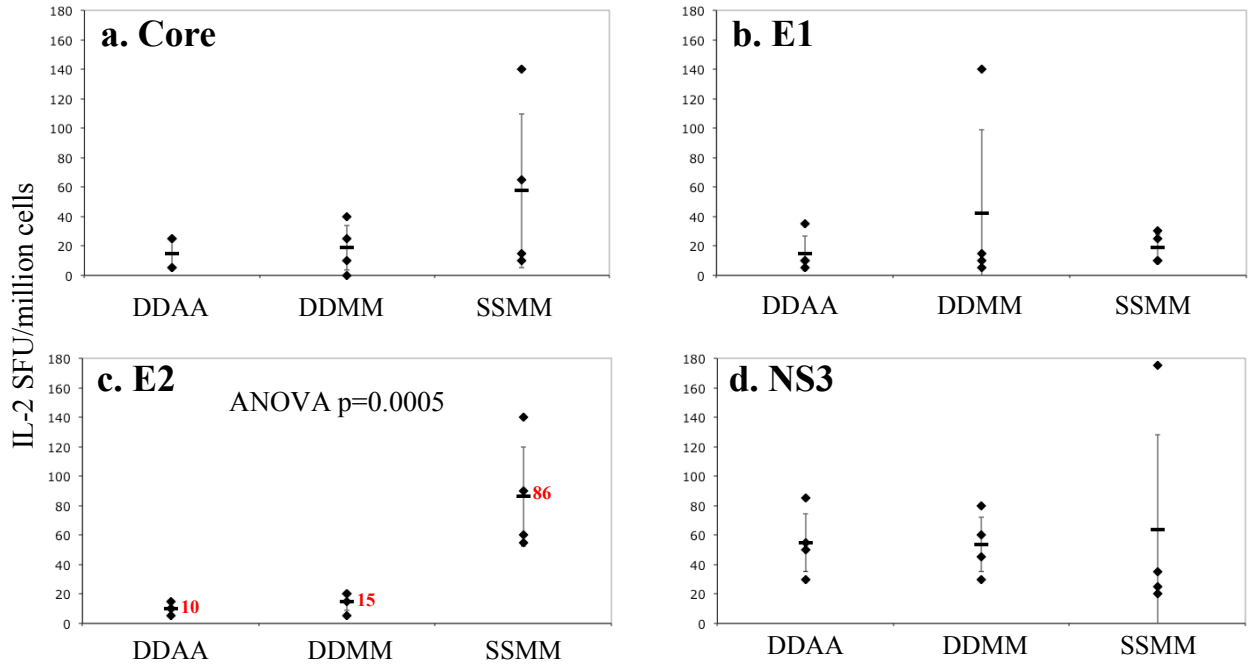
## Supplementary Figure 2

Individual IFN-gamma responses to each HCV antigen after Ad or MVA boost. Some individual (black) and average (red) values mentioned in the text are indicated for ease of reading.



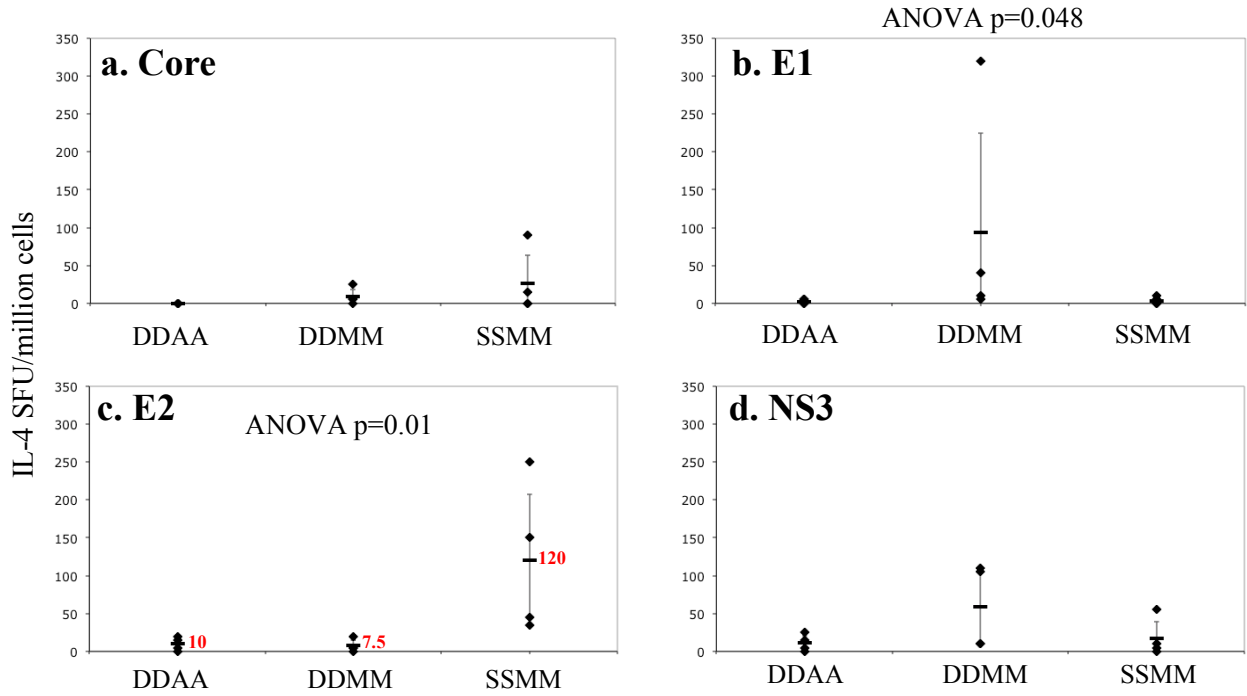
### Supplementary Figure 3

Individual IL-2 responses to each HCV antigen after Ad or MVA boost. Average values for E2 are indicated in red.



## Supplementary Figure 4

Individual IL-4 responses to each HCV antigen after Ad or MVA boost. Average values for E2 are indicated in red.



## Supplementary Figure 5

Sequence homology between the reagents used in the vectors and in the pseudoparticle assay, performed with CLUSTAL W (1.74) multiple sequence alignment. The HVR1 sequence is indicated with a black line.

```
GC1b  DLMGYIPLVGAPLGGAAARALAHGVRVLEdGVNYATGNLPGCSFSIFLLALLSCLTIPASA
1b J  DLMGYIPLVGAPLGGAAARALAHGVRVLEdGVNYATGNLPGCSFSIFLLALLSCLTIPASA
*****

GC1b  YEVRNVSGIYHVTNDCSNSSIVYEADMIMHTPGCVPCVRESNFSRCWVALTPTLAARNS
1b J  YEVRNVSGIYHVTNDCSNSSIVYEADMIMHTPGCVPCVRESNFSRCWVALTPTLAARNS
*****

GC1b  SIPTTTIRRHDLLVGAALCSAMYVGDLCGSVFLVSQLFTFSPRRYETVQDCNCSIYPG
1b J  SIPTTTIRRHDLLVGAALCSAMYVGDLCGSVFLVSQLFTFSPRRYETVQDCNCSIYPG
*****

GC1b  HVSGRMAWdMMMNWSPttALVVSQLLRIPQAVVDMVAGAHWGLAGLAYYSMVGNWAKV
1b J  HVSGRMAWdMMMNWSPttALVVSQLLRIPQAVVDMVAGAHWGLAGLAYYSMVGNWAKV|
*****

GC1b  LIVMLLFAGVDGHTHTVtGGRVASSTQSLVSWLSQGSPQKIQLVNTNGSWHINRTALNCND
1b J  LIVMLLFAGVDGHTHTVtGGRVASSTQSLVSWLSQGSPQKIQLVNTNGSWHINRTALNCND
*****

GC1b  SLQTGFIAALFYAHRFNASGCPERMASCRPIDKFAQGWGPITHVVPNISDQRPYCWHYAP
1b J  SLQTGFIAALFYAHRFNASGCPERMASCRPIDKFAQGWGPITHVVPNISDQRPYCWHYAP
*****:*****:*****

GC1b  QPCGIVPASQVCGPVYCFtPSPVVVGTDRSGVPTYSWGENETDVLllLNTRPPQGNWFG
1b J  QPCGIVPASQVCGPVYCFtPSPVVVGTDRFGAPTYSWGENETDVLllSNTRPPQGNWFG
:***** * ,*****

GC1b  CTWMNSTGFTKtCGGPPCNIGGVGNNTLICPTDCFRKHPEATYTKCGSGPWLTPRCLVDY
1b J  CTWMNSTGFTKtCGGPPCNIGGVGNNTLVCPTDCFRKHPEATYTKCGSGPWLTPRCMVDY
*****:*****:*****

GC1b  PYRLWHPCTINFTIFKVRMYVGGVEHRLNAACNWtRGERCDLEDRDRSELSPLllSTTE
1b J  PYRLWHPCTVNFtVFKVRMYVGGVEHRLNAACNWtRGERCDLEDRDRSELSPLllSTTE
*****:***:*****

GC1b  WQVLPCSFTtLPALSTGLIHLHQNIvDvQYLYGVGSVVVSVVikWEYVllLFLllADARV
1b J  WQILPCSFTtLPALSTGLIHLHRNIvDvQYLYIGSAVVVFAIKWEYIllLFLllADARV
** :*****:*****:***.***.*****

GC1b  CAclWmMllIAQAEA
1b J  CAclWmMllIAQAEA
*****
```